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CAP interacts with cytoskeletal proteins and regulates adhesion-mediated ERK activation and motility

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CAP/Ponsin belongs to the SoHo family of adaptor molecules that includes ArgBP2 and Vinexin. These proteins possess an N-terminal sorbin homology (SoHo) domain and three C-terminal SH3 domains that bind to diverse signaling molecules involved in a variety of cellular processes. Here, we show that CAP binds to the cytoskeletal proteins paxillin and vinculin. CAP localizes to cell–extracellular matrix (ECM) adhesion sites, and this process requires binding to vinculin. Overexpression of CAP induces the aggregation of paxillin, vinculin and actin at cell–ECM adhesion sites. Moreover, CAP inhibits adhesion-dependent processes such as cell spreading and focal adhesion turnover, whereas a CAP mutant that is unable to localize to cell–ECM adhesion sites is incapable of exerting these effects. Finally, depletion of CAP by siRNA-mediated knockdown leads to enhanced cell spreading, migration and the activation of the PAK/MEK/ERK pathway in REF52 cells. Taken together, these results indicate that CAP is a cytoskeletal adaptor protein involved in modulating adhesion-mediated signaling events that lead to cell migration.

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Introduction

Cell adhesion and motility are integrated processes that play crucial roles in a variety of physiological and pathophysiological functions (Webb *et al*, 2002; Ridley *et al*, 2003; Playford and Schaller, 2004; Raftopoulou and Hall, 2004). During embryonic development, cells migrate to the appropriate locations for terminal differentiation and tissue morphogenesis. In adults, cell motility is essential for homeostatic processes like wound healing and immune responses. Aberrant cell migration can lead to pathological conditions that include vascular or chronic inflammatory diseases, tumor invasion and metastasis.

The organization of the actin cytoskeleton and cell–extracellular matrix (ECM) adhesions are coordinately regulated during cell adhesion and migration. Integrins are the major transmembrane receptors for the ECM, and mediate the formation of cell adhesion structures by recruiting a characteristic set of proteins to their cytoplasmic regions (Hynes, 2002). More than 50 proteins have been found in cell–ECM adhesions, including scaffolding molecules, small GTPases, and enzymes such as kinases, phosphatases and proteases (Zamir and Geiger, 2001). While structural proteins like vinculin, talin and actinin function as physical links to the actin cytoskeleton, signaling molecules such as focal adhesion kinase (FAK) and paxillin transduce the stimuli from ECM to effectors that regulate adhesion formation and reorganize the actin cytoskeleton (Ridley *et al*, 2003).

The ERK/MAPK pathway regulates cell motility through its modulation of adhesion turnover (Huang *et al*, 2004; Webb *et al*, 2004). Activation of ERK at the cell periphery is required for adhesion disassembly, which favors cell spreading and migration in several cell types. Conversely, blockade of the pathway with the MEK inhibitors PD98059 and U0126, as well as with expression of dominant-negative mutants of MEK1 or ERK, inhibit cell migration induced by ECM. Two downstream effectors of ERK, calpain 2 and MLCK, are important for adhesion turnover (Huang *et al*, 2004). Calpain 2 is a calcium-dependent protease that is phosphorylated and activated by ERK at focal adhesion sites. This leads to the cleavage of FAK and other components of focal adhesions, and subsequent adhesion turnover (Carragher *et al*, 2003). At focal adhesions and at leading edge of migrating cells, ERK also phosphorylates and activates MLCK, which in turn phosphorylates MLC, leading to the severing and disassembly of focal adhesions (Klemke *et al*, 1997; Fincham *et al*, 2000; Webb *et al*, 2004). Disassembly of adhesions occurs at the cell rear and at the base of lamellipodia protrusions, allowing cells to continuously form forward protrusions at the leading edge and detach from the substrate at the trailing edge (Webb *et al*, 2002).

CAP, together with ArgBP2 and Vinexin (encoded by the genes *SORBS1*, *SORBS2* and *SORBS3*, respectively), belong to the SoHo family of adaptor proteins that are involved in the regulation of diverse cellular processes, including glucose transport, transcriptional activation and ubiquitination (Kioka *et al*, 2002). These proteins all have a sorbin peptide homology (SoHo) domain in the N-terminus, and three tandem SH3 domains in the C-terminus. We demonstrate

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here that CAP functions as a negative regulator of cell spreading and migration, suggesting an important role for CAP in the regulation of adhesion dynamics required for cell motility.

Results

CAP is localized at cell–ECM adhesions

The *SORBS1* gene that encodes CAP is expressed in numerous tissues (Wang *et al*, 1997; Ribon *et al*, 1998b; Kawabe *et al*, 1999; Kioka *et al*, 1999; Mandai *et al*, 1999; Lebre *et al*, 2001), and undergoes significant alternate splicing as has been described previously (Lin *et al*, 2001; Zhang *et al*, 2003; Alcazar *et al*, 2004; Matson *et al*, 2005). Western blot analysis with an anti-CAP antibody revealed numerous protein bands that were expressed at different levels in COS-1, CV-1, HeLa, NIH/3T3, REF52, and 3T3-L1 cells (Figure 1A).

We analyzed the subcellular localization of endogenous CAP in CV-1 and REF52 cells due to the high expression of the protein in these cell lines. CAP has been shown to localize at cell–cell and cell–matrix junctional complex regions in mouse mammary tumor MTD-1A cells and rat 3Y1 fibroblasts (Mandai *et al*, 1999). Similarly, immunofluorescence microscopy demonstrated that CAP co-localized with paxillin at cell–ECM adhesion sites in both CV-1 and REF52 cells (Figure 1B). Moreover, this colocalization is more striking at the peripheral adhesion sites compared to central ones. Double staining of the cells with an anti-CAP antibody and phalloidin revealed that CAP is localized at the ends of actin stress fibers, where F-actin is attached to cell adhesion structures (Figure 1C).

CAP interacts with paxillin and vinculin

Since we showed that endogenous CAP co-localizes with paxillin (Figure 1B), co-immunoprecipitation experiments were performed to further explore whether these proteins directly interact. CV-1 cell lysates were subjected to immunoprecipitation with an anti-CAP antibody or control rabbit IgG, followed by Western blot analysis. As shown in Figure 2A, paxillin was specifically co-immunoprecipitated with CAP. Consistent with our previous studies, endogenous vinculin also co-precipitated with CAP.

Paxillin has five leucine-rich motifs (LD repeats) in its N-terminal half, and four LIM domains in the C-terminal half of the molecule, which targets paxillin to focal adhesions. A short, proline-rich region in paxillin spanning amino acids 48–57 (PPPVPPPP), which lies between the first and second LD repeats, has been predicted as a potential binding motif for SH3-domain containing proteins (Brown and Turner, 2004). To delineate the CAP-binding site on paxillin, we generated paxillin mutants with either a truncation at the C-terminus to delete all the LIM domains (ΔLIM), or an internal deletion to remove the short proline-rich region at the N-terminal domain (ΔPro). GFP-tagged wild-type (WT) or mutant paxillin constructs were co-transfected into COS-1 cells with empty vector or myc-tagged CAP. Cells were lysed and immunoprecipitated with anti-myc antibodies. As shown in Figure 2B, both WT and ΔLIM GFP-paxillin co-immunoprecipitated with myc-CAP, whereas the ΔPro mutant of paxillin failed to bind, indicating that the proline-rich region mediates paxillin interaction with CAP, most likely through the SH3 domains of CAP.

Next, we set out to determine which SH3 domain(s) in CAP is responsible for this interaction. GST-fusion proteins containing all three SH3 domains (GST-ABC) or each individual SH3 domain of CAP were incubated with fibroblast cell lysates, and the precipitates were analyzed by Western blotting. Paxillin was pulled down by GST-SH3A and SH3B of CAP, but not GST-SH3C or GST alone (Figure 2C). Alternatively, FLAG-tagged WT and mutant (ΔPro) paxillin were *in vitro* translated, and incubated with the same GST-CAP SH3 domain fusion proteins, to demonstrate direct binding of WT, but not the ΔPro mutant to CAP SH3 domains (Supplementary Figure 1). Previous studies have shown that the first two SH3 domains of CAP are also responsible for the interaction of the protein with vinculin (Mandai *et al*, 1999). We confirmed and extended these findings, showing that vinculin was precipitated by SH3B and to a much lesser extent, SH3A (Figure 2C).

To verify the specificity of binding of the SH3 domains of CAP to paxillin and vinculin, we introduced point mutations into CAP to disrupt the function of each SH3 domain. Tryptophan residues at position 536, 610 or 716 in the SH3 domains were mutated to phenylalanine (WF mutants).

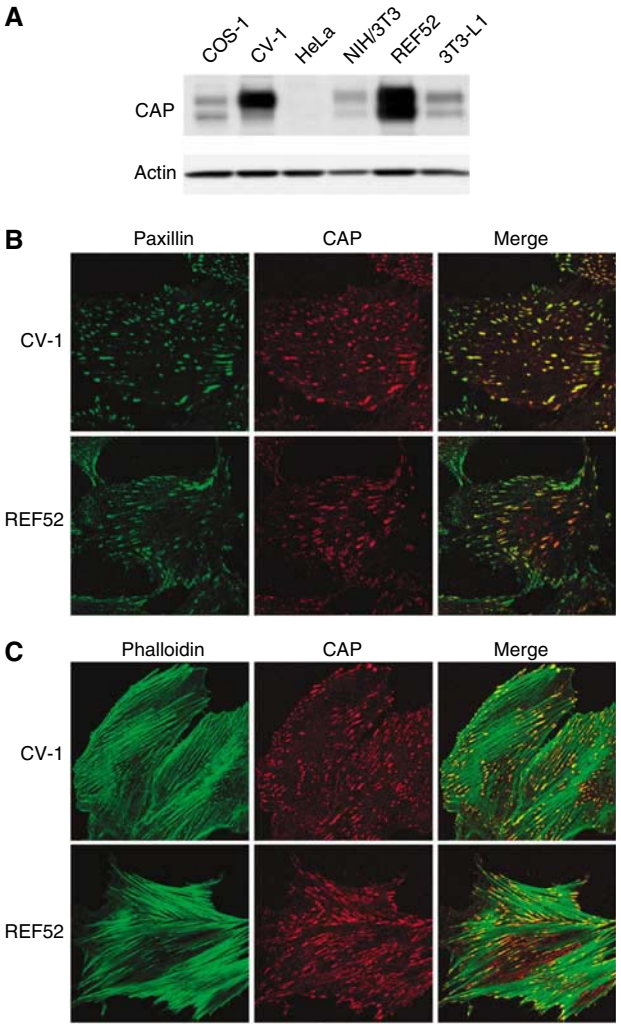

Figure 1 Expression and subcellular localization of endogenous CAP. (**A**) Western blot of CAP protein levels in various cell lines. (B, C) Immunofluorescence of CAP (red) in CV-1 and REF52 cells, (**B**) co-stained with paxillin (green) to demonstrate colocalization of CAP with paxillin at cell–ECM adhesions; (**C**) co-stained with phalloidin (green) to show the localization of CAP at the ends of actin stress fibers.

These residues are highly conserved in most SH3 domains and are required for binding to proline-rich sequences (Lim and Richards, 1994). Myc-tagged WT or WF mutants of CAP were co-transfected into COS-1 cells with GFP-paxillin and vinculin. Cell lysates were immunoprecipitated with an anti-myc antibody, and analyzed by Western blotting. As shown in Figure 2D, GFP-paxillin was co-precipitated with WT myc-CAP, as well as with CAP bearing a mutation in its third SH3 domain. In contrast, W/F substitutions in the first or second SH3 domain decreased the binding to paxillin. The co-precipitation of CAP with vinculin exhibited a similar pattern; mutants of the first and the second SH3 domains of CAP exhibited dramatically decreased binding to vinculin. W/F substitutions were also created in both the first and second SH3 domains together ($W^{536/610}F$). This compound mutant (referred to as W2F) was completely unable to interact with either paxillin or vinculin. Taken together, these data demonstrated that the first two SH3 domains of CAP are required for its interaction with both paxillin and vinculin.

Vinculin recruits CAP to cell–ECM adhesions

Since the W2F mutant of CAP is unable to bind to either paxillin or vinculin, we examined its localization by immunofluorescence microscopy. Myc-tagged WT CAP or its W2F mutant were transiently transfected into CV-1 cells, which were then co-stained with anti-paxillin and anti-myc antibodies (Figure 3A). WT myc-CAP co-localized with paxillin at cell–ECM adhesion sites, whereas the W2F mutant exhibited plasma membrane localization along with a diffused fibrillar distribution throughout the cytoplasm. These results indicate

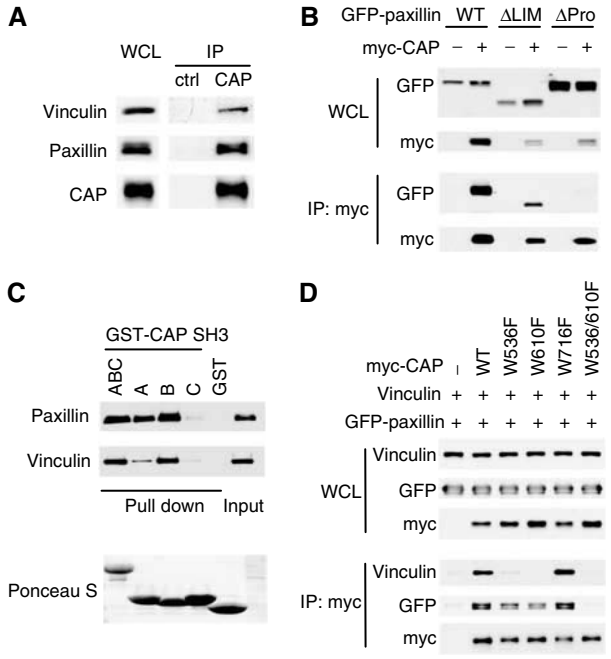

Figure 2 The first two SH3 domains of CAP mediate its interaction with both paxillin and vinculin. (**A**) Co-immunoprecipitation of endogenous paxillin and vinculin with CAP in CV-1 cells. (**B**) CAP interacts with paxillin through a proline-rich region. COS-1 cells were co-transfected with empty vector or myc-CAP and GFP-paxillin WT, ΔLIM or ΔPro constructs. Cells were lysed and immunoprecipitated with anti-myc antibody, and the coprecipitated paxillin was detected by Western blot analysis. (**C**) Paxillin and vinculin bind to the first two SH3 domains of CAP. GST-fusion proteins containing all three SH3 domains (GST-CAP SH3 ABC) or each individual SH3 domain of CAP were used to pull down paxillin and vinculin from fibroblast lysates. GST alone was used as a control. The bottom panel shows the loading of the GST fusion proteins. (**D**) Point mutations in CAP SH3 domains diminished its binding to paxillin and vinculin. COS-1 cells were co-transfected with GFP-paxillin, vinculin and WT or mutants of myc-CAP. Lysates were immunoprecipitated with anti-myc antibody, and co-precipitated GFP-paxillin and vinculin were detected by Western blot.

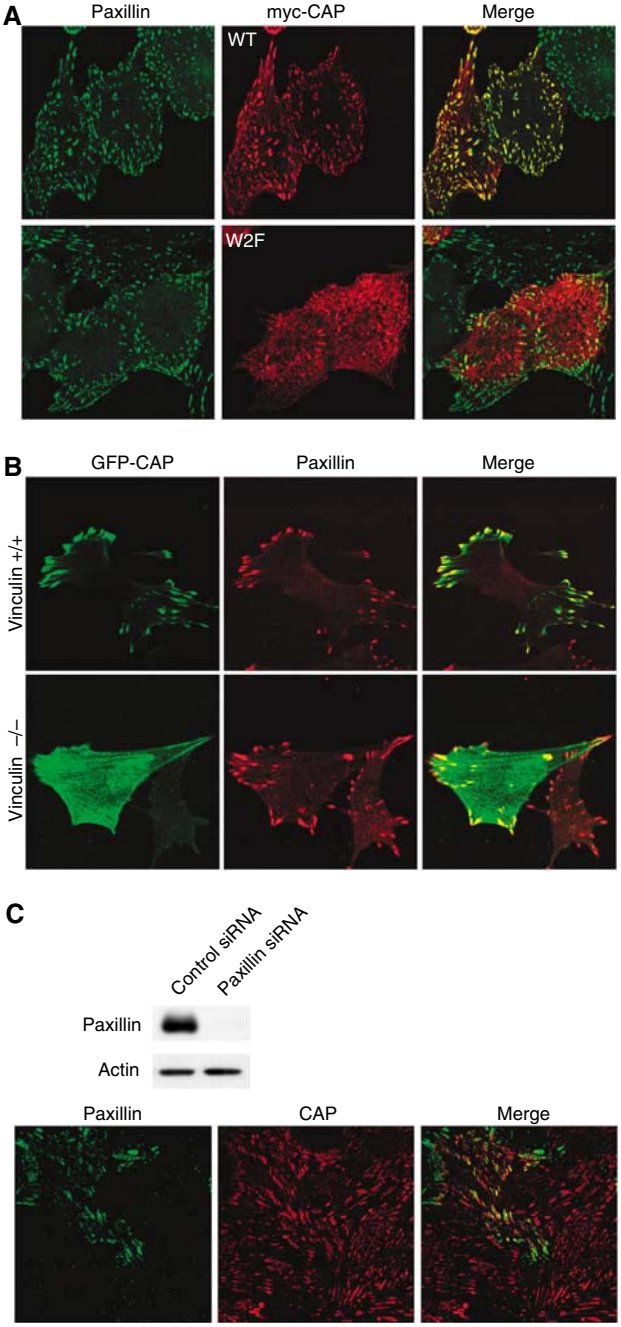

Figure 3 Vinculin recruits CAP to cell–ECM adhesions. (**A**) CV-1 cells were transiently transfected with myc-tagged WT CAP or the W2F mutant, and co-stained with anti-paxillin (green) and anti-myc (red) antibodies. (**B**) WT (vinculin+/+) or vinculin-null (vinculin−/−) MEFs were transfected with GFP-CAP, and stained with anti-paxillin antibody. (**C**) REF52 cells were transfected with scrambled control siRNA or paxillin siRNA, lysed and subjected to Western blotting for paxillin expression; actin was used as a loading control. Paxillin siRNA transfected cells were co-stained with anti-paxillin (green) and anti-CAP (red) antibodies.

that the first two SH3 domains are required for the localization of CAP to cell–ECM adhesions.

While both paxillin and vinculin interact with the SH3 domains of CAP, which protein anchors CAP to cell–ECM adhesions remains unknown. We examined the localization of CAP in vinculin-null fibroblasts. WT or vinculin-null mouse embryonic fibroblasts (MEFs) were transfected with GFP-CAP, and stained with an anti-paxillin antibody. As expected, GFP-CAP co-localized with paxillin at cell–ECM adhesions in WT (vinculin+/+) fibroblasts. In contrast, in vinculin-null cells (vinculin−/−), GFP-CAP showed a diffused and fibrillar distribution with slight accumulation at cell–ECM adhesions (Figure 3B). Paxillin localization remained at cell–ECM adhesions regardless of vinculin expression. On the other hand, siRNA-mediated knockdown of paxillin in REF52 cells did not affect the focal adhesion localization of CAP. As shown in Figure 3C, CAP remains at cell–ECM adhesion sites regardless of the presence of paxillin. Vinculin localization did not change in paxillin knockdown cells (data not shown). These results indicate that vinculin is more crucial for the recruitment of CAP to cell–ECM adhesion sites.

CAP induces actin, paxillin and vinculin aggregation at cell–ECM adhesions

Next, we investigated the function of CAP in the organization of focal adhesions and the actin cytoskeleton. COS-1 cells were transfected with the myc-tagged WT or W2F mutant of CAP followed by immunofluorescence staining with an anti-myc antibody and phalloidin to visualize myc-CAP and actin. Overexpression of WT CAP induced a coalescence of F-actin into short aggregates. CAP co-localized with actin at these aggregates (Figure 4A). In contrast, the W2F mutant failed to induce actin aggregation at cell–ECM adhesions, but did co-localize well with actin at the plasma membrane and in cortical stress fibers (Figure 4A). These data suggest that CAP re-organizes F-actin at cell–ECM adhesions.

The effect of CAP on adhesion structures was similarly evaluated by co-staining COS-1 cells overexpressed myc-CAP with an anti-paxillin antibody. As shown in Figure 4B, overexpression of WT CAP induced the aggregation of paxillin and the enlargement of cell–ECM adhesion structures. Conversely, the W2F mutant was without effect. Vinculin staining showed a similar pattern (data not shown).

To determine whether CAP induced a redistribution of paxillin and vinculin to actin cytoskeletal structures, cell lysates were separated into Triton X-100 soluble and insoluble fractions. The amounts of vinculin and paxillin were determined by Western blotting of the detergent insoluble fraction, which contains the cytoskeleton and cell–ECM adhesion complexes. As shown in Figure 4C, overexpression of WT CAP, but not the W2F mutant, increased the amount of vinculin and paxillin in the Triton X-100-insoluble fraction by approximately two-fold.

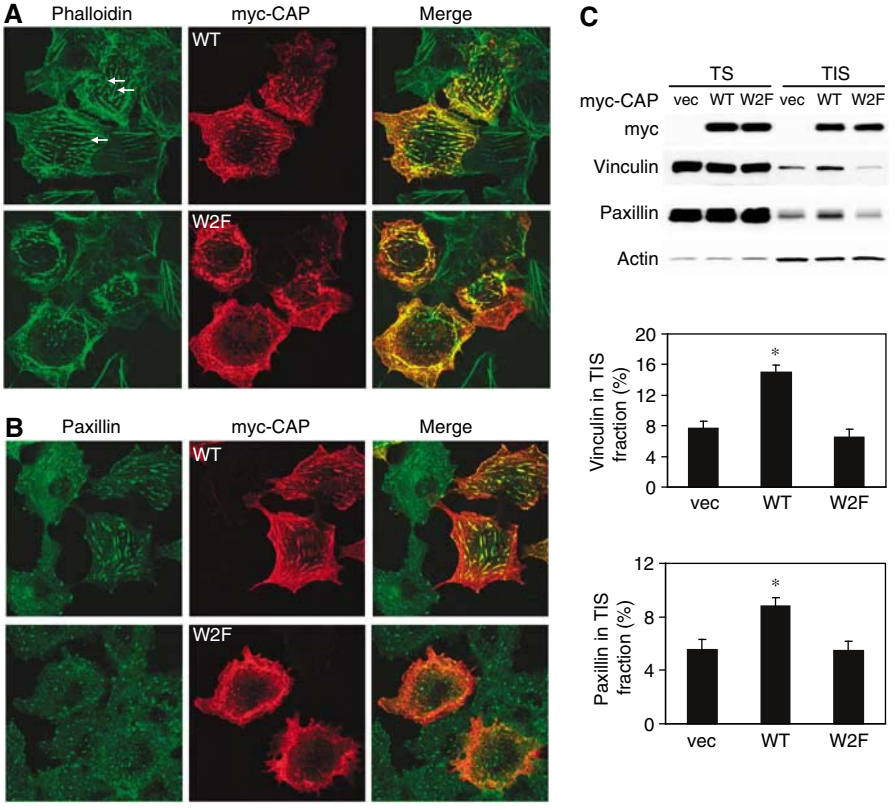

Figure 4 CAP induces actin, paxillin and vinculin aggregation at cell–ECM adhesions. (A, B) COS-1 cells were transfected with myc-tagged WT CAP or the W2F mutant, and co-stained with (**A**) phalloidin (green) and anti-myc (red) antibody, or (**B**) anti-paxillin (green) and anti-myc (red) antibodies. Arrows indicate actin aggregates at the ends of stress fibers. (**C**) COS-1 cells were transfected with either empty vector, myc-tagged WT CAP or the W2F mutant. Triton-soluble (TS) and -insoluble (TIS) fractions were separated as described in Materials and methods. Equal amount of proteins were loaded on a SDS–PAGE gel, and the levels of vinculin and paxillin in each fraction were detected by Western blot. Actin was blotted as a loading control. The graphs on the bottom show the quantitation of the percentage of vinculin or paxillin in triton-insoluble fractions from six experiments. The data represent mean±s.e. $^*P<0.05$.

CAP impairs focal adhesion turnover

The enlarged cell–ECM adhesion structures induced by overexpression of CAP suggest a role for CAP in modulating adhesion turnover. It has previously been shown that treatment of cells with nocodazole, followed by a washout period that allows the disrupted microtubules to regrow, induces the transient disassembly of cell–ECM adhesions (Ezratty *et al*, 2005). We utilized this assay to study the effect of CAP expression on adhesion turnover under these conditions. CV-1 cells were transiently transfected with myc-CAP, serum starved and treated with nocodazole for 4 h. Nocodazole was then washed out and cells were fixed at different time points for immunofluorescence microscopy. Cell–ECM adhesions were visualized by staining with an anti-paxillin antibody. As shown in Figure 5A, cell–ECM adhesions in untransfected cells were stabilized by nocodazole treatment, and then underwent disassembly for up to 60 min after nocodazole washout (indicated by asterisks). Overexpression of myc-CAP prevented the turnover of adhesions (indicated by arrows). In contrast, the W2F mutant failed to stabilize cell–ECM adhesions in this assay (Figure 5B). These data suggest that CAP inhibits cell–ECM adhesion disassembly, and further that the focal adhesion localization of CAP is important for its role in regulating focal adhesion turnover.

CAP negatively regulates cell spreading and migration

Since regulated assembly and disassembly of focal adhesions is fundamental to several adhesion dependent processes, we decided to investigate the involvement of CAP in the regulation of cell spreading and migration. COS-1 cells were transiently transfected with GFP, GFP-CAP WT or W2F mutant, and subsequently re-plated on fibronectin for 10, 20 or 40 min. Over 90% of cells were transfected when examined by fluorescence microscopy (data not shown). Overexpression of WT CAP inhibited cell spreading on fibronectin at each time point (Figure 6A). At 20 min after plating on fibronectin, 58% of the GFP transfected cells spread, whereas only 34% of GFP-CAP transfected cells exhibited spread morphology (Figure 6B). The W2F mutant of CAP

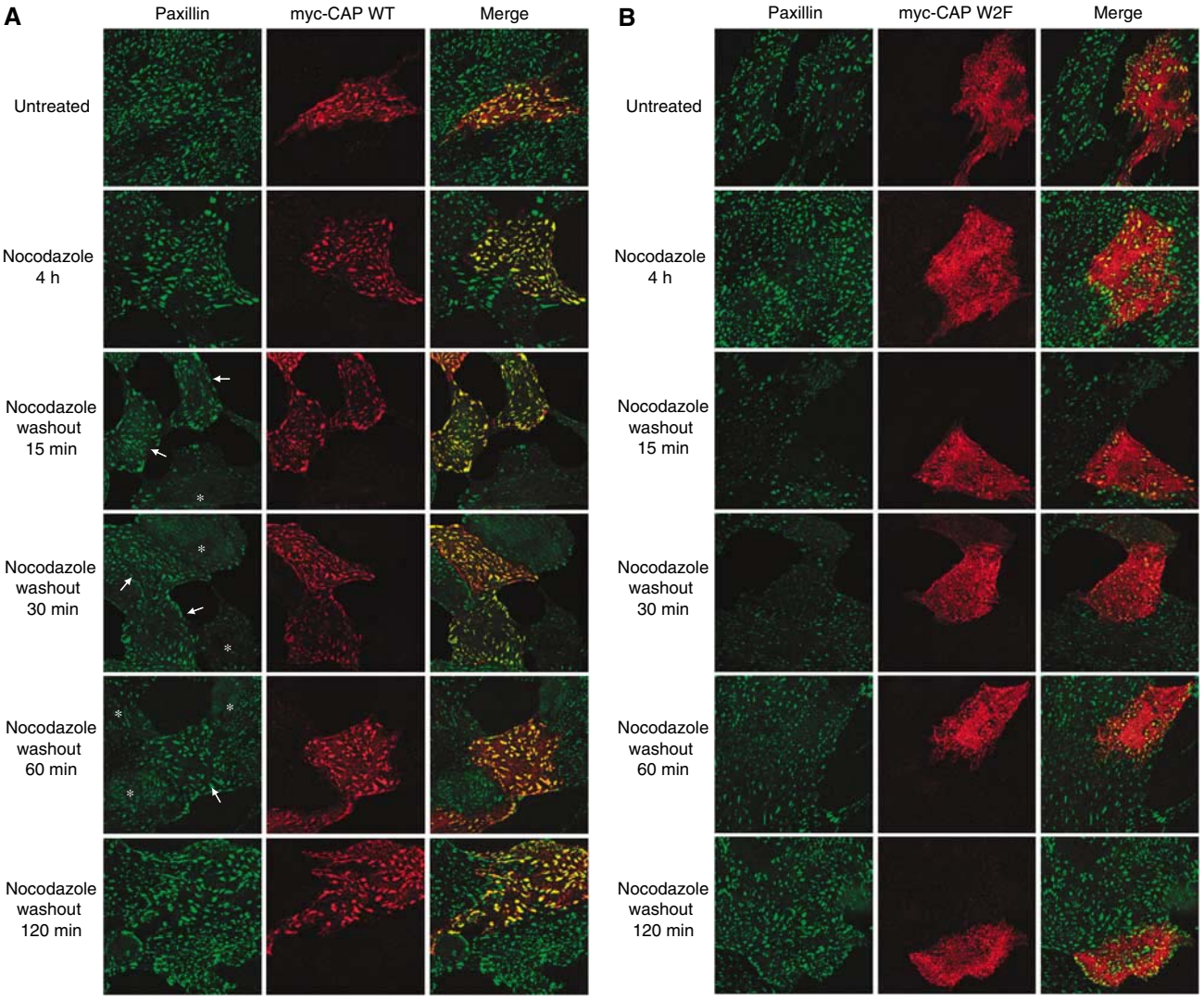

Figure 5 Overexpression of WT CAP, not the W2F mutant, impairs focal adhesion turnover. CV-1 cells on coverslips were transfected with myc-CAP (**A**) or myc-CAP W2F mutant (**B**). Cells were starved overnight, and then left untreated, or treated with 10 μM nocodazole for 4 h. Nocodazole was then washed out and cells were fixed at the indicated time points, and co-stained with anti-paxillin (green) and anti-myc (red) antibodies. In (A), cells expressing myc-CAP are indicated by arrows, and the ones not transfected are indicated by asterisks.

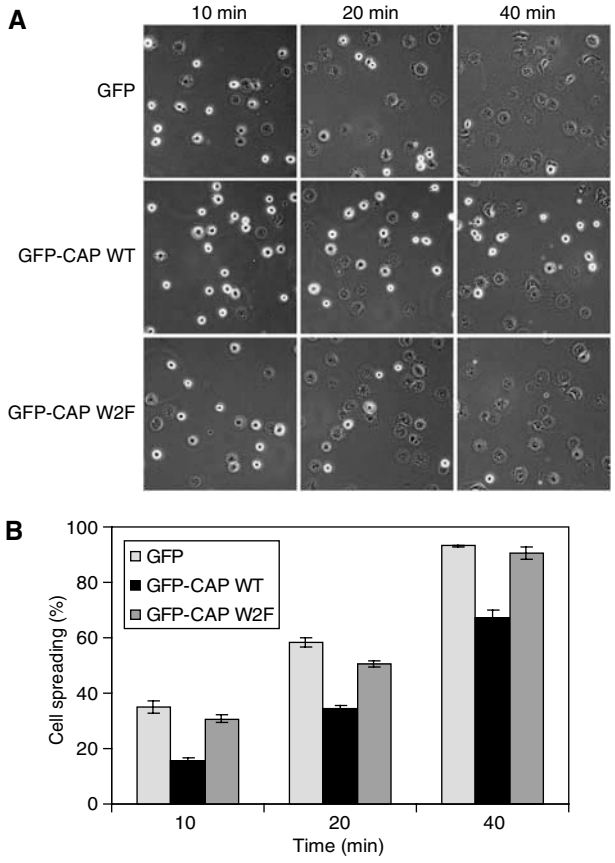

Figure 6 Overexpression of WT CAP, not the W2F mutant, inhibits cell spreading on fibronectin. COS-1 cells were transfected with either GFP control, GFP-CAP WT or the W2F mutant. Cells were than plated on fibronectin-coated plates and pictures were taken at indicated times. A representative field of each time points was shown (**A**), and the percentage of spread cells was calculated from five fields (**B**). The data represent mean ± s.e.

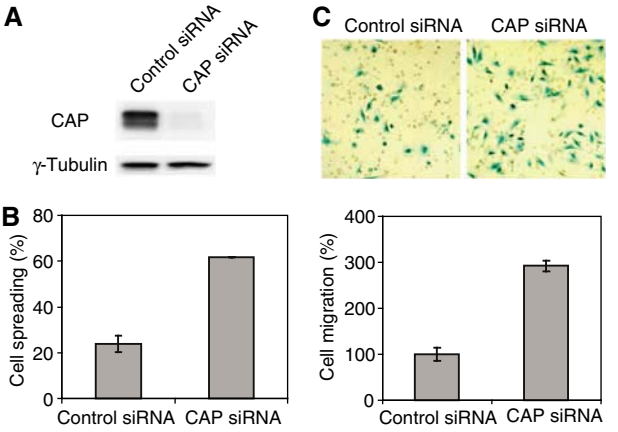

Figure 7 Knockdown of CAP in REF52 fibroblasts promotes cell spreading and migration. (**A**) Cells were transfected with scrambled control siRNA or CAP siRNA, lysed and subjected to Western blotting for CAP expression; γ-tubulin was used as a loading control. (**B**) Cells were plated on fibronectin-coated plates for 20 min, photographed and the percentage of spread cells was counted. (**C**) Equal number of REF52 cells transfected with the indicated siRNA were seeded on Transwell filters coated on both sides with fibronectin. The cells were allowed to migrate through the filters toward fibronectin (10 μg/ml) in the lower chamber for 5 h. Migrated cells were stained with methylene blue and photographed. The representative pictures were shown on the top. The bar graph on the bottom shows the relative number of migrated cells counted from three filters, where the number of control cells was set to 100%. The data represent mean ± s.e.

only exhibited a mild inhibitory effect at early time points (10 and 20 min), indicating that localization to cell–ECM adhesions is important for the ability of CAP to regulate cell spreading.

We further investigated the effect of reducing CAP expression on cell spreading and motility by siRNA-mediated knockdown in REF52 fibroblasts. Western blot analysis demonstrated efficient (>90%) depletion of CAP protein by treatment of cells with siRNA, compared to the control scrambled siRNA (Figure 7A). Three days after transfection of oligos, when efficient knockdown was observed, cells were collected and plated on fibronectin. At 20 min after plating, 24% of control cells spread. In contrast, 62% of cells that were deficient in CAP spread at the same time point (Figure 7B). Taken together these data suggest that CAP expression negatively regulates cell spreading on fibronectin.

To explore the role of CAP in cell motility, modified Boyden chamber migration assays were performed. Control or CAP knockdown cells were allowed to migrate towards 10 μg/ml fibronectin for 5 h. Migrated cells were stained with methylene blue, photographed and quantitated. As shown in Figure 7C, CAP-deficient cells migrated nearly three-fold more efficiently than did control cells, suggesting that CAP functions to negatively influence cell motility.

To further confirm the results obtained from siRNA-mediated knockdown experiments, we isolated CAP-deficient primary MEFs from E13 embryos (Liesnewski *et al*, submitted). Western blot analysis confirmed the deletion of CAP in the knockout cell lines. As shown in Supplementary Figure 2A, CAP null MEF cells exhibited improved spreading on fibronectin, demonstrating that loss of CAP is sufficient to induce spreading of primary fibroblasts.

Depletion of CAP enhances fibronectin-mediated ERK activation

Integrin engagement to ECM is known to activate several signaling molecules involved in cell migration including ERK, PAK and FAK. Thus, we tested whether CAP regulates these pathways by examining adhesion-mediated signaling in CAP-deficient REF52 cells. A significant increase in ERK phosphorylation was observed upon re-plating cells onto fibronectin in CAP-deficient compared to control cells (Figure 8A). Elevated ERK phosphorylation was also observed in CAP-deficient MEF cells (Supplementary Figure 2B). These data suggest that CAP negatively regulates activation of the MAP kinase pathway during adhesion to fibronectin.

We further explored the upstream signaling events that may lead to this enhanced activation of ERK. Phosphorylation of MEK was also significantly increased in CAP knockdown compared to control cells (Figure 8A). Adhesion to the ECM has been reported to activate the Ras→Raf→MEK→ERK signaling pathway. One way this occurs is through the phosphorylation of FAK at Tyr^{925}, which recruits Grb2 and SOS to activate Ras, leading to the activation of ERK (Schlaepfer *et al*, 1994). However, no significant differences were observed in phosphorylation of

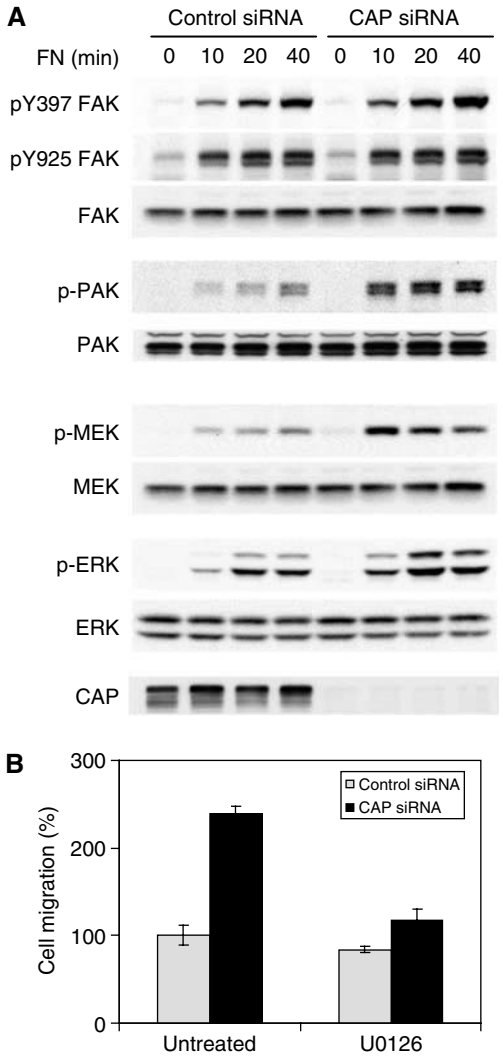

Figure 8 CAP regulates adhesion-dependent ERK signaling. (**A**) Knockdown of CAP in REF52 cells enhanced fibronectin-stimulated PAK/MEK/ERK phosphorylation. Cells were kept in suspension or plated on fibronectin for the indicated times. Lysates were separated by SDS–PAGE and subjected to Western blot analysis with the indicated antibodies. (**B**) Inhibition of ERK decreased cell migration in CAP knockdown REF52 cells. Modified Boyden chamber assays were performed in the absence or presence of 10 μM U0126. The data represent mean ± s.e.

FAK at Tyr^{397} (autophosphorylation site) or Tyr^{925} (Grb2 binding site) between control and CAP knockdown cells (Figure 8A).

Numerous studies have also demonstrated that phosphorylation of PAK plays an important role in adhesion-induced activation of Raf and MEK. Interestingly, siRNA-mediated knockdown of CAP resulted in a marked increase of PAK phosphorylation (Figure 8A). Taken together, these data suggested that CAP negatively regulates adhesion induced ERK activation through the PAK/MEK/ERK pathway.

Finally, to further verify the role of ERK in the regulation of cell motility by CAP, we examined the effect of CAP depletion on cell migration in the presence of the MEK inhibitor U0126. Modified Boyden chamber assays were performed as described above. As shown in Figure 8B, U0126 inhibited the increase in REF52 cell migration induced by CAP knockdown, confirming that changes in the MAP kinase pathway are required for the regulation of cell motility by CAP.

Discussion

The SoHo family of proteins is comprised of CAP, Vinexin and ArgBP2. These proteins exhibit a similar architecture, with three C-terminal SH3 domains and a region of similarity to the peptide sorbin (SoHo domain) in the N-terminus (Kioka *et al*, 2002). The SoHo proteins interact with different cytoskeletal or signaling molecules (Wang *et al*, 1997; Ribon *et al*, 1998b; Kawabe *et al*, 1999; Kioka *et al*, 1999; Mandai *et al*, 1999; Baumann *et al*, 2000; Soubeyran *et al*, 2003; Zhang *et al*, 2003; Haglund *et al*, 2004; Cestra *et al*, 2005), and have been implicated in a variety of cellular processes including insulin-stimulated glucose transport (Baumann *et al*, 2000). We demonstrate here that CAP localizes at cell–ECM adhesions, and plays a crucial role in regulating several adhesion-dependent events.

CAP colocalizes with and binds to the cytoskeletal proteins paxillin and vinculin, both of which are known to play important roles in adhesion dynamics and cell motility. The interactions, which occur at cell–ECM adhesions, require the first two SH3 domains of CAP and the proline-rich motifs of paxillin and vinculin. At the same time, vinculin can bind directly to paxillin LD motifs (Brown and Turner, 2004). Deletion of the paxillin proline-rich motif, which presumably is not required for vinculin binding, resulted in the complete loss of CAP association, indicating that the CAP–paxillin interaction is not bridged through the binding of these proteins to vinculin. Moreover, CAP directly binds to paxillin *in vitro*, demonstrating that this interaction is not mediated through other proteins that may associate with the proline-rich motif of paxillin such as Src (Weng *et al*, 1993).

While both paxillin and vinculin interact with CAP, studies using vinculin-null fibroblasts and paxillin-depleted REF52 cells demonstrate that vinculin, rather than paxillin, is primarily responsible for recruiting CAP to focal adhesions. Interestingly, a recent study indicated that the adhesion targeting of another SoHo family member, Vinexinβ, is also mediated by vinculin (Chen *et al*, 2005).

The N-terminal region of CAP also binds to actin (Supplementary Figure 3). This may explain the fibrillar localization of the W2F mutant and the localization of CAP in vinculin-null cells. Although the W2F mutant does not localize to focal adhesions or affect their stability, it may retain some function in organizing actin fibers, since this mutant form of CAP can still interact with actin. In this regard, overexpression of the W2F mutant in COS-1 cells induces the formation of cortical actin stress fibers (Figure 4A), and mildly inhibits cell spreading (Figure 6). The direct interaction between CAP and F-actin, and the biological functions of this interaction require more study.

The enlarged focal adhesions caused by ectopic expression of CAP are reminiscent of those found in FAK-null fibroblasts, which exhibit decreased migration and spreading (Ilic *et al*, 1995). Similarly, we show here by several criteria that CAP functions as a negative regulator of cell spreading and migration (Figures 6 and 7; Supplementary Figure 2). While overexpression of CAP impeded spreading of COS-1 cells on fibronectin, spreading of REF52 fibroblasts was enhanced by siRNA-mediated knockdown of endogenous

CAP. Knockdown of CAP in REF52 cells also resulted in increased migration in a chemotaxis assay with fibronectin as stimulus.

We further note that in both REF52 cells depleted of CAP and CAP-null MEF cells, there was a significant increase in adhesion-stimulated ERK activation. This is in agreement with increased cell motility observed in these cells, since ERK is known to promote cell spreading and migration by facilitating focal adhesion turnover (Huang *et al*, 2004; Webb *et al*, 2004). In this regard, overexpression of WT CAP was able to block microtubule-induced cell–ECM adhesion disassembly during a nocodazole washout experiment (Figure 5). These data reveal a unique function of CAP in the stabilization of adhesion complexes and the resultant prevention ;of cell migration, likely through attenuation of ERK activity. Indeed, the specific MEK inhibitor U0126 diminished the effect of CAP depletion on cell migration (Figure 8B), confirming the involvement of ERK MAPK cascade in this function of CAP.

Integrin engagement initiates multiple signaling cascades that lead to ERK activation. Adhesion stimulates the autophosphorylation of FAK at Tyr^{397}, creating a binding site for the SH2 domain of c-Src. This leads to enhanced phosphorylation of FAK at other sites, including Tyr^{925}, which creates a binding site for Grb2, and linkage to the ERK/MAPK pathway (Mitra *et al*, 2005). Additionally, several studies have suggested an important role for PAK in anchorage-dependent signal transduction leading to activation of the ERK/MAPK pathway (Frost *et al*, 1997; Howe and Juliano, 2000; del Pozo *et al*, 2000). Adhesion to fibronectin induces PAK1-dependent phosphorylation of MEK1 on S^{298}, a phosphorylation event necessary for activation of MEK1 and MAPK (Slack-Davis *et al*, 2003). PAK has also been reported to phosphorylate Raf-1 on S^{338}, increasing its catalytic activity (King *et al*, 1998; Sun *et al*, 2000). We show here that depletion of CAP resulted in a dramatic increase in adhesion-induced PAK phosphorylation, while FAK activity was unaffected (Figure 8A).

Recent studies indicate that the MEK scaffolding proteins MP1 and p14 regulate adhesion-dependent ERK activation through a PAK1-dependent pathway (Pullikuth *et al*, 2005). Furthermore, both MP1 and p14 transiently inhibit Rho/ROCK function, which are necessary for adhesion turnover and cell spreading. Rho family small GTPases play critical roles in the regulation of cytoskeleton dynamics and cell motility (Raftopoulou and Hall, 2004). While Rho activity is required to induce actin–myosin contractility during cell migration, Rho may also negatively regulate cell migration by inducing excessive contractile forces and focal adhesions (Cox *et al*, 2001). Moreover, PAK has been suggested to serve an adaptor function in stimulating the transition of Rho focal adhesions to Cdc42/Rac focal adhesions to facilitate directional motility (Brown *et al*, 2002). Interestingly, we observed that endogenous CAP co-localizes better with focal adhesions at cell periphery. Therefore, CAP may be involved in the spatial-temporal regulation of Rho activity through the PAK and/or ERK pathways. It will be interesting to determine whether CAP affects all or only a subset of these pathways.

Finally, our studies demonstrated that the localization of CAP to focal adhesions and its interaction with paxillin and vinculin are important for its functions. The W2F mutant of CAP failed to induce the aggregation of cytoskeletal proteins at cell–ECM adhesions (Figure 4C) and their relocalization to triton-insoluble fractions. Moreover, this mutant cannot stabilize focal adhesions (Figure 5B), and failed to block cell spreading (Figure 6).

Many proteins involved in the regulation of ECM–cell adhesion dynamics are also involved in the regulation of cell proliferation, differentiation and survival (Hynes, 2002). We have previously shown that stable cell lines overexpressing CAP exhibit a reduced growth rate (Ribon *et al*, 1998a). Increased CAP expression correlates with the differentiation of adipocytes (Ribon *et al*, 1998b) and myoblasts (unpublished data), suggesting a role for CAP in the induction and maintenance of the differentiated phenotype. Additionally, CAP protein appears to be downregulated in transformed COS-1 cells. Therefore, we speculate that CAP may also function at a convergent point of adhesion and growth factor signaling pathways in the control of cell proliferation and differentiation. Current studies are focused on addressing these potential functions of CAP.

Materials and methods

Antibodies and reagents

CAP (E-20), GFP (FL), Myc (9E10) and PAK (N-20) antibodies were purchased from Santa Cruz Biotechnology. β-Actin and γ-tubulin antibodies were obtained from Sigma. Vinculin and another CAP antibody were from Upstate Biotechnology, Inc. Paxillin antibodies were purchased from BD Biosciences. Phospho-FAK (Tyr397) antibodies were from BioSource. The phospho-FAK (Tyr925), phospho-PAK1/2, phospho-MEK1/2, phospho-ERK, total MEK1/2 and total ERK antibodies were from Cell Signaling Technology. The Alexa Fluor secondary antibodies and phalloidin were from Molecular Probes. Nocodazole and human plasma fibronectin were purchased from Sigma. Protein A/G-agarose beads were from Santa Cruz Biotechnology.

Plasmids and mutagenesis

Myc- and glutathione *S*-transferase (GST)-tagged CAP constructs were previously described (Ribon *et al*, 1998b; Zhang *et al*, 2003). GFP-CAP was constructed by subcloning CAP cDNA in frame in the *Bam*HI/*Eco*RI sites of the eGFP-C1 vector (BD Biosciences). All point mutations of CAP were generated using the Stratagene Quick Change mutagenesis kit, according to the manufacturer's protocol. The vinculin cDNA was kindly provided by Dr Eileen D Adamson. The GFP-paxillin construct was kindly provided by Dr Christopher E Turner. The GFP-paxillin(ΔLIM) mutant was generated by inserting a stop codon before the LIM domains of WT paxillin using PCR. The GFP-paxillin(ΔPro) mutant was generated by PCR-mediated internal deletion. All mutations and cloning products were confirmed by sequencing.

Cell culture and transient transfection

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The vinculin-null fibroblasts were kindly provided by Dr Eileen D Adamson. To obtain primary embryonic fibroblasts, WT and CAP-null E13 embryos were isolated. The head and organs were removed, and the remaining carcasses were minced and dispersed into single-cell suspensions in trypsin at 37°C for 30 min. Afterwards, cells were resuspended in medium and plated, and experiments were performed within three passages. COS-1 cells were transfected using FuGene 6 reagent (Roche), according to the manufacturer's instructions. Other transfections were done using Lipofectamine 2000 (Invitrogen).

siRNA knockdown

The expression of CAP in REF52 cells was inhibited using Stealth RNAi from Invitrogen. REF52 cells were transfected with CAP-specific siRNA duplexes, or the scrambled oligos as control, using Lipofectamine 2000, following the manufacturer's protocol. Two targeted sequences that were proven to effectively mediate the silencing of CAP expression are as follows: 5′-GCACAGGACCUAAG

CAGUGUGUCUA-3′, and 5′-GGUGAUACACAAGUAGAAAUGUCUU-3′. After transfection, cells were cultured for 72 h before subjected to Western blot, spreading or migration assays. The sequence of the siRNA oligo used for targeting paxillin is 5′-GAAGAGAUUGGA UCCCGGAACUUCU-3′.

Immunoprecipitation and immunoblotting

For immunoprecipitation studies, cells were washed twice with ice-cold phosphate-buffered saline and were lysed for 30 min at 4°C with buffer containing 50 mM Tris–HCl (pH 8.0), 135 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 mM NaF, and protease inhibitors (Roche). Clarified lysates were incubated with the indicated antibodies for 2 h at 4°C. Immune complexes were precipitated with protein A/G-agarose for 1 h at 4°C and were washed extensively with lysis buffer before solubilization in SDS sample buffer. Bound proteins were resolved by SDS–PAGE and transferred onto nitrocellulose membranes. Individual proteins were detected with the specific antibodies and visualized by blotting with horseradish peroxidase-conjugated secondary antibodies.

FN-mediated ERK activation

Serum-starved cells were collected by trypsinization, washed, and then kept in suspension at 37°C for 1 h. Afterwards, cells were lysed immediately or replated onto tissue culture dishes precoated with fibronectin (BD Biosciences) for the indicated times. For these studies, cells were lysed in RIPA buffer (above lysis buffer including 0.5% sodium deoxycholate and 0.1% SDS).

GST pull-down assay

GST-CAP fusion proteins were expressed in the *Escherichia coli* strain BL21 and purified as described previously (Liu and Brautigan, 2000). Fibroblast cells were lysed as described above for immunoprecipitation. Lysates were incubated either with GST alone or with GST-CAP variants immobilized on glutathione-Sepharose beads (Amersham Pharmacia) for 1 h at 4°C. The beads were washed extensively with lysis buffer, and the bound proteins were solubilized in SDS sample buffer and analyzed by immunoblotting. In a similar assay, FLAG-tagged WT and mutant paxillin were generated by coupled *in vitro* transcription/translation (TNT; Promega), diluted in the lysis buffer, and subjected to the pull-down assay.

Confocal fluorescence microscopy

Cells were grown on glass coverslips in six-well dishes. Following the fixation with 10% formalin for 20 min, cells were permeabilized with 0.5% Triton X-100 for 5 min and then blocked with 1% bovine serum albumin, 1% ovalbumin and 2% goat serum for 1 h. Coverslips were incubated with 2 μg/μl primary and Alexa Fluor secondary antibodies in blocking solution, and mounted on glass slides with Vectashield (Vector Laboratories). Cells were imaged using confocal fluorescence microscope (Olympus IX SLA). Images were then imported into Photoshop (Adobe Systems, Inc.) for processing.

Triton X-100 soluble and insoluble fractionation

Cells were washed with cell solubilization buffer (CSB) containing 10 mM PIPES, 50 mM KCl, 10 mM EGTA, 3 mM $MgCl_2$, 2 M glycerol, 2 mM NaF, 1 mM Na_3VO_4 and protease inhibitors, then incubated for exactly 5 min at 4°C in CSB containing 1% Triton X-100. This soluble fraction was collected, and the plates were washed once with CSB, the remaining cytoskeletal fraction was lysed in extraction buffer containing 20 mM Tris–HCl, 300 mM NaCl, 30 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and protease inhibitors. The triton-insoluble fraction was passed through a 28-gauge syringe 10 times before protein quantification and Western blot analysis.

Actin co-sedimentation assays

GST-CAP fusion proteins were prepared as previously described (Liu and Brautigan, 2000), and eluted from the beads with GST elution buffer (20 mM glutathione, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl). Fusion proteins were dialyzed against PBS/10% glycerol for 16 h at 4°C. Actin co-sedimentation assays were performed using an Actin Binding Protein Spin-Down Assay Kit (Cytoskeleton, Inc.), according to the manufacturer's description. The supernatant and pellet fractions were analyzed by SDS–PAGE, transferred onto nitrocellulose membrane and visualized with Ponceau S (Sigma).

Cell spreading assay

Serum-starved cells were collected by trypsinization, washed, counted and resuspended in DMEM. Cells were kept in suspension for 1 h, and then 5×10^5 cells were added to 35-mm tissue culture dishes that were precoated with fibronectin (BD Biosciences). Cells were allowed to spread for the indicated times at 37°C, chilled on ice for 10 min, and then photographed. Spread cells were defined as cells with extended processes, lacking a rounded morphology and not phase-bright, whereas non-spread cells were rounded and phase-bright under microscope. Three random microscopic fields were counted per plate, and all experiments were repeated three times.

Cell motility assay

Cell migration was determined using a modified Boyden chamber assay. Both sides of the transwell membrane (tissue culture-treated, 6.5-mm diameter, 8-μm pores; Becton Dickinson Labware) were coated with fibronectin (10 μg/ml) for 1 h at 37°C. Cells were starved, trypsinized and washed twice with DMEM. 1×10^5 cells were added to the upper chamber, and the lower chamber was filled with DMEM containing 10 μg/ml of fibronectin. When the MEK inhibitor was used in this assay, cells were treated with 10 μM of U0126 for 30 min before trypsinization, and also added into both the upper and lower chambers during migration. After incubation at 37°C for 5 h, the membranes were fixed in 10% formalin, and cells on the upper surface were mechanically removed with cotton swabs. Migrated cells on the lower side of membranes were stained with methylene blue and photographed. Three random microscopic fields were counted per well, and all experiments were performed in triplicate.

Focal adhesion disassembly assay

Serum-starved CV-1 cells were treated with 10 μM nocodazole for 4 h to completely depolymerize microtubules. The drug was washed out with serum-free medium, and allowed to recover for different intervals of time. Cells were then fixed in 10% formalin for 20 min and subjected to immunofluorescence microscopy.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Alcazar O, Ho RC, Fujii N, Goodyear LJ (2004) cDNA cloning and functional characterization of a novel splice variant of c-Cbl-associated protein from mouse skeletal muscle. *Biochem Biophys Res Commun* **317:** 285–293

Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, Bickel PE, Pessin JE, Saltiel AR (2000) CAP defines a second signalling pathway required for insulin-stimulated glucose transport (see comments). *Nature* **407:** 202–207

Brown MC, Turner CE (2004) Paxillin: adapting to change. *Physiol Rev* **84:** 1315–1339

Brown MC, West KA, Turner CE (2002) Paxillin-dependent paxillin kinase linker and p21-activated kinase localization to

focal adhesions involves a multistep activation pathway. *Mol Biol Cell* **13:** 1550–1565

Carragher NO, Westhoff MA, Fincham VJ, Schaller MD, Frame MC (2003) A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. *Curr Biol* **13:** 1442–1450

Cestra G, Toomre D, Chang S, De Camilli P (2005) The Abl/Arg substrate ArgBP2/nArgBP2 coordinates the function of multiple regulatory mechanisms converging on the actin cytoskeleton. *Proc Natl Acad Sci USA* **102:** 1731–1736

Chen H, Cohen DM, Choudhury DM, Kioka N, Craig SW (2005) Spatial distribution and functional significance of activated vinculin in living cells. *J Cell Biol* **169:** 459–470

Cox EA, Sastry SK, Huttenlocher A (2001) Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. *Mol Biol Cell* **12:** 265–277

del Pozo MA, Price LS, Alderson NB, Ren XD, Schwartz MA (2000) Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. *EMBO J* **19:** 2008–2014

Ezratty EJ, Partridge MA, Gundersen GG (2005) Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol* **7:** 581–590

Fincham VJ, James M, Frame MC, Winder SJ (2000) Active ERK/MAP kinase is targeted to newly forming cell–matrix adhesions by integrin engagement and v-Src. *EMBO J* **19:** 2911–2923

Frost JA, Steen H, Shapiro P, Lewis T, Ahn N, Shaw PE, Cobb MH (1997) Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *EMBO J* **16:** 6426–6438

Haglund K, Ivankovic-Dikic I, Shimokawa N, Kruh GD, Dikic I (2004) Recruitment of Pyk2 and Cbl to lipid rafts mediates signals important for actin reorganization in growing neurites. *J Cell Sci* **117:** 2557–2568

Howe AK, Juliano RL (2000) Regulation of anchorage-dependent signal transduction by protein kinase A and p21-activated kinase. *Nat Cell Biol* **2:** 593–600

Huang C, Jacobson K, Schaller MD (2004) MAP kinases and cell migration. *J Cell Sci* **117:** 4619–4628

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110:** 673–687

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377:** 539–544

Kawabe H, Hata Y, Takeuchi M, Ide N, Mizoguchi A, Takai Y (1999) nArgBP2, a novel neural member of ponsin/ArgBP2/vinexin family that interacts with synapse-associated protein 90/postsynaptic density-95-associated protein (SAPAP). *J Biol Chem* **274:** 30914–30918

King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, Marshall MS (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature* **396:** 180–183

Kioka N, Sakata S, Kawauchi T, Amachi T, Akiyama SK, Okazaki K, Yaen C, Yamada KM, Aota S (1999) Vinexin: a novel vinculin-binding protein with multiple SH3 domains enhances actin cytoskeletal organization. *J Cell Biol* **144:** 59–69

Kioka N, Ueda K, Amachi T (2002) Vinexin, CAP/ponsin, ArgBP2: a novel adaptor protein family regulating cytoskeletal organization and signal transduction. *Cell Struct Funct* **27:** 1–7

Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresh DA (1997) Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* **137:** 481–492

Lebre AS, Jamot L, Takahashi J, Spassky N, Leprince C, Ravise N, Zander C, Fujigasaki H, Kussel-Andermann P, Duyckaerts C, Camonis JH, Brice A (2001) Ataxin-7 interacts with a Cbl-associated protein that it recruits into neuronal intranuclear inclusions. *Hum Mol Genet* **10:** 1201–1213

Lim WA, Richards FM (1994) Critical residues in an SH3 domain from Sem-5 suggest a mechanism for proline-rich peptide recognition. *Nat Struct Biol* **1:** 221–225

Lin WH, Chiu KC, Chang HM, Lee KC, Tai TY, Chuang LM (2001) Molecular scanning of the human sorbin and SH3-domain-containing-1 (SORBS1) gene: positive association of the T228A polymorphism with obesity and type 2 diabetes. *Hum Mol Genet* **10:** 1753–1760

Liu J, Brautigan DL (2000) Insulin-stimulated phosphorylation of the protein phosphatase-1 striated muscle glycogen-targeting subunit and activation of glycogen synthase. *J Biol Chem* **275:** 15940–15947

Mandai K, Nakanishi H, Satoh A, Takahashi K, Satoh K, Nishioka H, Mizoguchi A, Takai Y (1999) Ponsin/SH3P12: an l-afadin- and vinculin-binding protein localized at cell–cell and cell–matrix adherens junctions. *J Cell Biol* **144:** 1001–1017

Matson SA, Pare GC, Kapiloff MS (2005) A novel isoform of Cbl-associated protein that binds protein kinase A. *Biochim Biophys Acta* **1727:** 145–149

Mitra SK, Hanson DA, Schlaepfer DD (2005) Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* **6:** 56–68

Playford MP, Schaller MD (2004) The interplay between Src and integrins in normal and tumor biology. *Oncogene* **23:** 7928–7946

Pullikuth A, McKinnon E, Schaeffer HJ, Catling AD (2005) The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals. *Mol Cell Biol* **25:** 5119–5133

Raftopoulou M, Hall A (2004) Cell migration: Rho GTPases lead the way. *Dev Biol* **265:** 23–32

Ribon V, Herrera R, Kay BK, Saltiel AR (1998a) A role for CAP, a novel, multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions. *J Biol Chem* **273:** 4073–4080

Ribon V, Printen JA, Hoffman NG, Kay BK, Saltiel AR (1998b) A novel, multifuntional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 adipocytes. *Mol Cell Biol* **18:** 872–879

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR (2003) Cell migration: integrating signals from front to back. *Science* **302:** 1704–1709

Schlaepfer DD, Hanks SK, Hunter T, van der Geer P (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372:** 786–791

Slack-Davis JK, Eblen ST, Zecevic M, Boerner SA, Tarcsafalvi A, Diaz HB, Marshall MS, Weber MJ, Parsons JT, Catling AD (2003) PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. *J Cell Biol* **162:** 281–291

Soubeyran P, Barac A, Szymkiewicz I, Dikic I (2003) Cbl-ArgBP2 complex mediates ubiquitination and degradation of c-Abl. *Biochem J* **370:** 29–34

Sun H, King AJ, Diaz HB, Marshall MS (2000) Regulation of the protein kinase Raf-1 by oncogenic Ras through phosphatidylinositol 3-kinase, Cdc42/Rac and Pak. *Curr Biol* **10:** 281–284

Wang B, Golemis EA, Kruh GD (1997) ArgBP2, a multiple Src homology 3 domain-containing, Arg/Abl-interacting protein, is phosphorylated in v-Abl-transformed cells and localized in stress fibers and cardiocyte Z-disks. *J Biol Chem* **272:** 17542–17550

Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol* **6:** 154–161

Webb DJ, Parsons JT, Horwitz AF (2002) Adhesion assembly, disassembly and turnover in migrating cells—over and over and over again. *Nat Cell Biol* **4:** E97–E100

Weng Z, Taylor JA, Turner CE, Brugge JS, Seidel-Dugan C (1993) Detection of Src homology 3-binding proteins, including paxillin, in normal and v-Src-transformed Balb/c 3T3 cells. *J Biol Chem* **268:** 14956–14963

Zamir E, Geiger B (2001) Components of cell–matrix adhesions. *J Cell Sci* **114:** 3577–3579

Zhang M, Kimura A, Saltiel AR (2003) Cloning and characterization of Cbl-associated protein splicing isoforms. *Mol Med* **9:** 18–25